POLYPHOSPHATE AFFECTS KEY METABOLIC PROCESSES IN *DICTYOSTELIUM DISCOIDEUM*

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Polyphosphate Affects Key Metabolic Processes in *Dictyostelium discoideum*

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The goal of this project is to determine how physiological levels of polyphosphate affect cellular metabolism in the model organism *Dictyostelium discoideum*. Understanding the effects of polyphosphate on a molecular level is important, as its activity in *Dictyostelium* pathways is likely analogous to its activity in human macrophages, and thus polyphosphate shows great promise as a target for human therapeutics in a variety of diseases. In order to quantify the interactions of polyphosphate with *Dictyostelium* metabolism, I examined its effect on pseudopod formation, mitochondrial size and mass, ATP levels, cellular oxygen consumption, intracellular glucose content, and expression of the genes Plc, Pten, Grld, IplA, and PiaA. The results demonstrate that polyphosphate impedes pseudopod formation in wild type AX2 *Dictyostelium* cells. Additionally, polyphosphate increases mitochondrial size and mass in *Dictyostelium* cells and influences the expression of all genes examined. Polyphosphate seemed to induce a small increase in intracellular ATP levels, but the results were not significant. No significant impact on glucose content and oxygen consumption were observed. Ultimately, polyphosphate may interact with *Dictyostelium* metabolism through the affecting mitochondrial processes and gene expression.

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SECTION I

INTRODUCTION

Dictyostelium discoideum as a model organism

Dictyostelium discoideum is a powerful tool for studying cellular processes from basic metabolic pathways to host cell-pathogen interactions. A model eukaryotic organism, *Dictyostelium* is a social amoeba with a well-understood growth curve and physiology. These amoebas also closely resemble human immune cells including neutrophils and macrophages. *Dictyostelium* begins its humble life on a plate as a single celled organism in the vegetative state and then proceeds through a log phase, slug phase, and fruiting body in order to complete its life cycle as a multicellular aggregate organism. The stages of development are spurred both by nutrient availability and secreted signals like cAMP. For instance, secreted cAMP signals cells to aggregate and alters gene expression so that cells begin to form differentiated multicellular structures [1]. Additionally, other secreted factors like polyphosphate regulate cell proliferation and viability as they are produced in response to increasing cell density and other factors [2]. With an easily manipulated genome and wide variety of knockout and rescue mutants, the effects of important genes can be studied at every stage of development. The similarity between *Dictyostelium discoideum* and human macrophages allows us to easily study many immune responses and disorders at a molecular level.

Polyphosphate in biological systems

Polyphosphate is an ancient molecule found in all kingdoms of life. It is comprised of five to hundreds of inorganic phosphate residues linked by high energy phospho-anhydride

bonds [3]. Polyphosphate has been proposed to govern many aspects of biological systems, from the development of early life to enabling pathogenicity.

Roles of polyphosphate in bacteria

In bacteria, Polyphosphate kinase 1 (ppk1) is mainly responsible for polyphosphate synthesis. Ppk1 hydrolyzes ATP to add a phosphate residue onto a nascent polyphosphate chain. Since the reaction is reversible, it can also proceed in the opposite direction to use polyphosphate as a substrate for ATP synthesis. In prokaryotes, polyphosphate is most notably linked to its roles in bacterial survival and virulence in *Mycobacterium tuberculosis*, *Neisseria meningitidis, Helicobacter pylori, Vibrio cholerae, Salmonella typhimurium, Shigella flexneri, Pseudomonas aeruginosa, Bordetella pertussis, Yersinia pestis*, and many more notorious pathogens [4]. In such species, polyphosphate is a necessary component of the stringent response, survival of oxidative stress, survival in the stationary phase, motility, and the degradosome [4]. When Ppk1 was knocked out in *Mycobacterium tuberculosis* bacteria, they displayed reduced survival in the stationary phase and in nitrosative stress conditions as well as decreased resistance to macrophages compared to wild type tuberculosis [5]. Since macrophages are the body's first line of defense against bacteria like tuberculosis, it is possible that the bacteria secrete polyphosphate when they infect humans in order to survive inside the macrophage, in a way enjoying a free ride into the lungs. Given the absence of a ppk1 homolog in higher order eukaryotes, ppk1 may help us further understand host-pathogen interactions in human cells.

Roles of polyphosphate in human tissues

In addition to its many functions in prokaryotes, polyphosphate also regulates essential processes in mammals, including humans. For instance, polyphosphate has been identified as a potential regulator of bone calcification through inhibiting the formation and dissolution of

calcium phosphate crystals [6]. Humans also have the protein H-prune, our own form of exopolyphosphatase [7], which has been found to fuel metastasis in cancers by increasing tumor cell motility [8]. Finally, polyphosphate is also a component of the human inflammatory response through potentiating platelet activation and vascular hyperpermeability via Tor pathways [9] as well as upregulating expression of inflammatory cytokines in macrophages [10]. These factors strengthen the hypothesis that polyphosphate and its associated proteins are promising targets for therapeutics against inflammatory diseases and pathogens in human medicine.

Polyphosphate research in *Dictyostelium discoideum*

The goal of my experiments is to use the interaction between *Dictyostelium* and added polyphosphate to clarify the molecular mechanisms behind how bacteria like *Mycobacterium tuberculosis* might similarly use polyphosphate to thwart the human immune system. Additionally, my experiments may yield valuable info into how *Dictyostelium discoideum* and other eukaryotic cells use endogenous polyphosphate as a regulatory molecule. Therefore, it is necessary to elucidate both the effects of polyphosphate as an autocrine signal used by the *Dictyostelium* cells and as a factor secreted by pathogens to inhibit macrophage activity. *Dictyostelium discoideum* is a useful tool for studying polyphosphate because it is one of the few eukaryotes to possess a Ppk1 homolog, and it uses secreted polyphosphate in autocrine regulation. The presence of polyphosphate also affects the ability of *Dictyostelium* to phagocytose bacteria [11]. Such phagocytosis is naturally observed in normal feeding habits since bacteria like E. coli are staples in the amoeba's diet. However, polyphosphate allows some bacteria like *Salmonella Typhimurium* to survive inside *Dictyostelium* cells [11]. Polyphosphate and inositol phosphates also regulate key processes in *Dictyostelium,* including programmed cell

death, cell differentiation and other aspects of the cell cycle, cell aggregation, and proliferation [12].

Polyphosphate signaling and pathways

A hypothesis common to many *Dictyostelium* researchers is that polyphosphate exercises its effects in the amoeba through regulating gene expression and acting as signaling molecules in various pathways. Some polyphosphate signaling pathways have already been identified in D. discoideum. In a Ras/Akt pathway, proteins Ras and Akt mediate the ability of polyphosphate to induce development of *Dictyostelium* cells into the multicellular stages of their life cycle by increasing expression of a cell-cell adhesion molecule and promoting cell aggregation [3]. A negative feedback pathway relies on polyphosphate to regulate cell density in *Dictyostelium* cells [2]. Constitutively-secreted polyphosphate binds the cell surface and causes the rate of proliferation to decrease, and it even halts cell proliferation when added to cultures growing at mid-log phase [2]. In the identified programmed cell death pathway, inositol phosphates, which are important factors in polyphosphate synthesis, cause cell death in a dose and time-dependent manner as well as increase the expression of apoptotic factors [13]. In some experiments, high nutrient conditions can override the effects of polyphosphate, such as using 100% HL5 media over 25% HL5 media [14], which indicates that secreted polyphosphate may behave like a pre-starvation factor when cells become over-crowded and nutrient-deprived.

Key pathway components

The genes Phospholipase C (Plc), Phosphatase and tensin homolog (Pten), Glutamate receptor-like protein D (Grld), Inositol 1,4,5-trisphosphate receptor-like protein A (IplA), and Protein pianissimo A (PiaA) were identified as possible polyphosphate pathway components through screening mutant *Dictyostelium* cells for resistance to polyphosphate-induced

proliferation inhibition. Each of these genes has an important function in cell metabolism, biosynthesis, or the cell cycle. Three of these genes control a pathway initiated by cAMP to produce inositol1,4,5-trisphosphate (IP3) and dicaylglygerol (DAG). Pten converts Phosphatidylinositol 1,4,5-triphosphate (PIP3) to Phosphatidylinositol 4,5-bisphosphate (PIP2) while Plc cleaves PIP2 into IP3 and DAG [15]. IP3 subsequently activates the calcium channel IplA. Grld has been identified as a putative G protein-coupled receptor that mediates sensing of and response to polyphosphate in *D. discoideum* [14]. PiaA, an important cytoplasmic signal, is involved in development and through enabling activation of adenylate cyclase-coupled GPCR's [16]. Ultimately, I show that polyphosphate may exercise its regulatory effects in *Dictyostelium* using the interplay between these genes and other metabolic processes.

SECTION II

METHODS

Cell culture

Dictyostelium cells were grown in shaking culture and maintained at concentrations of about $2x10^6$cells/mL in HL5 (Formedium Ltd, Norwich, England) media with streptomycin. In all experiments, cells were treated with either 150 μM polyphosphate (Spectrum, New Brunswick, NJ) or an equivalent amount of pbm buffer (20 mM KH_2PO_4, 0.01 mM $CaCl_2$, 1 mM $MgCl_2$, pH 6.1) in the control group. The same stock of polyphosphate was used for all experiments.

Pseudopod formation assays

After 24 hr treatment with pbm or 150 μM poly p, a Nikon Ti2 microscope was used to record pseudopod formation in *Dictyostelium* cells. Cells at a density of $0.2x10^6$ cells/mL were allowed to adhere to a cover glass for 30 minutes, and then transferred to an Insall chamber with a uniform media gradient so that any chemotaxis effects would not be observed. Then, using the 20x objective, images were auto-captured every 2 seconds for 5 minutes. Imagej (NIH, Bethesda, MD) software was used to document every pseudopod produced by at least 10 cells per video. Cells that remained round (formed zero pseudopods), divided, or left the field of view during the duration of the video were not counted.

Mitochondrial assays

Wild type AX2 *D. discoideum* cultured as previously described were treated with 150 μM polyphosphate or an equivalent amount of pbm buffer for 24 hours. Then, to avoid polyphosphate interfering with the dye uptake as was previously observed, $1x10^6$ cells were

centrifuged at 1000 x g for 3 min, quickly washed with ice cold pbm buffer, and then resuspended in ice cold pbm. The cells were kept on ice for 5 minutes prior to dye addition. After 5 minutes, Mitotracker green dye (Thermofisher, Waltham, MA) was added to each tube at a concentration of 1 μM. The final conditions were 1x106 cells/mL with 1 μM dye. Cells were incubated on ice with the dye for 30 minutes. After 30 minutes, each tube was centrifuged at 1000 x g for 3 minutes, quickly washed twice with ice cold pbm and then resuspended in 500 μL pbm. Fla-1, Fsc-A and Ssc-A measurements for about 10,000 cells per sample in a standardized size/granularity range were then taken by a flow cytometer.

Extracellular oxygen consumption assay

The rate of oxygen consumption was measure using an Abcam oxygen consumption assay (Abcam, Cambridge, United Kingdom). The assay was conducted in triplicate to n=4 using a 96-well plate. After treatment with pbm or poly P for 24 hours, cells at a concentration of $4x10^6$ cells/mL were spun down at 1000 x g for 3 minutes, washed with fresh HL5 media, and then resuspended in HL5 + pbm or 150 μM poly p. 150 μL of cells were added to each well, and then the oxygen consumption probe was added. Probe fluorescence was measured over 90 minutes at an excitation wavelength of 620 nm and an emission wavelength of 380 nm using a plate reader. The slope of the trendline for average fluorescence/time was used to determine the oxygen consumption rate.

Intracellular ATP assay

Each ATP assay was performed in triplicate due to previously observed inconsistency/over-sensitivity within the assay. For each replicate, ATP was extracted from $1x10^6$ cells that had been treated with either 150 μM poly p or pbm for 24 hours using a boiling water method [17] with a few modifications. $1x10^6$ cells were centrifuged at 1000 x g for 3

minutes and washed with pbm to remove extracellular ATP from the samples. Then, the cell pellets were treated with 1 mL boiling sterile water and placed on a heating block at 100°C for 10 min to ensure thorough extraction. After 10 minutes of heating, tubes were vortexed to lyse the cells and then centrifuged at 12,000 rpm for 5 min at -4°C. The supernatant of each sample was transferred to a new tube for use in the assay. Any inconsistencies in volume were measured and adjusted for in calculating ATP concentration. A firefly luciferase ATP determination kit from ThermoFisher Scientific (Thermofisher, Waltham, MA) was used for all experiments, including generation of a new ATP standard curve for each experiment. Luminescence was measured using a 96 well plate with 10 µL supernatant or ATP standard and 100 µL of reaction standard solution from the kit. Measurements were taken on a plate reader at an excitation of 560 nm at room temperature.

Glucose content assay

The same set of cells was used for the glucose and protein assays in order to calculate glucose content. After 24 hours of treatment with pbm or poly p, 10 $x10^6$ - 15 $x10^6$ cells were taken from each group and then clarified as previously described. After being frozen at -20 °C for at least 2 hours, the cells were thawed and thoroughly vortexed to ensure lysis. The cell lysate was centrifuged at 12000 x g for 3 minutes for clarification, and the supernatant was used in a glucose assay kit from Abcam (Abcam, Cambridge, United Kingdom). A standard curve was prepared using known glucose concentrations. Fluorescence was measured in a 96-well plate using a plate reader.

cDNA synthesis and qPCR

mRNA was extracted from *Dictyostelium* cells after 0, 1, 4, and 8 hours treatment with pbm or 150 µM poly p using a ZYMO Research RNA Miniprep kit (Zymo Research, Irvine,

CA). $3x10^6 – 5x10^6$ cells were used from each sample. Then, the amount of RNA per sample was quantified for each sample, and cDNA was synthesized using reagents from the ZYMO kit. cDNA was stored at -80 °C until qPCR was performed in triplicate in a 96-well plate using the forward and reverse primers of the desired genes as well as GAPDH as a control. Relative expression levels were quantified and normalized no added polyphosphate at t=0.

SECTION III

RESULTS

Polyphosphate decreases pseudopod formation and increases pseudopod lifespan in *D. discoideum*

Replicates were done up to n=4, at which point significant differences in both pseudopod lifespan and the number of pseudopods formed per minute were observed between the control and poly p groups. For counting purposes, pseudopods were defined as non-filipodia projections from the main body of the cell. Every pseudopod formed over the 5-minute period was analyzed for least 10 cells per treatment group to attain the results for each replicate. Pseudopod lifespan quantification began when the pseudopod became distinguishable from the main body of the cell and ended when the pseudopod was no longer distinguishable from the main body of the cell. Cells treated with poly p ultimately displayed longer pseudopod lifespans than cells in the control group. The average pseudopod lifespan for a cell treated with Poly P was 47.76 seconds while the average pseudopod lifespan for a cell treated with pbm was 35.99 seconds (figure 1). In each replicate, the average pseudopod lifespan was lower in the control group than in the poly p group, indicating that the trend is consistent.

Pseudopod location was not expected to change in response to polyphosphate since a uniform concentration that should not affect chemotaxis was present. Location was defined by forming a figurative “X” over the screen of the laptop. Any pseudopod formed toward the top or bottom of the “X” was defined as being on the side, any pseudopod formed toward the left side of the “X” was on the back, and any pseudopod formed on the right side of the “X” was on the front. While the poly p cells grew fewer pseudopods in each location than the pbm cells, the ratio of pseudopods formed to the front, back, and side of the cell was about the same. In each group,

the cells grew about half as many pseudopods to the front and back as they did to the sides, indicating accurate and reliable counting methods.

Finally, the number of pseudopods formed per minute was calculated by dividing the total number of pseudopods formed on each cell by 5. Cells treated with poly p grew significantly fewer pseudopods per minute than cells in the control group. While pbm cells produced on average 6.51 pseudopods per minute, cells treated with poly p only produced an average of 4.45 pseudopods per minute (figure 1)

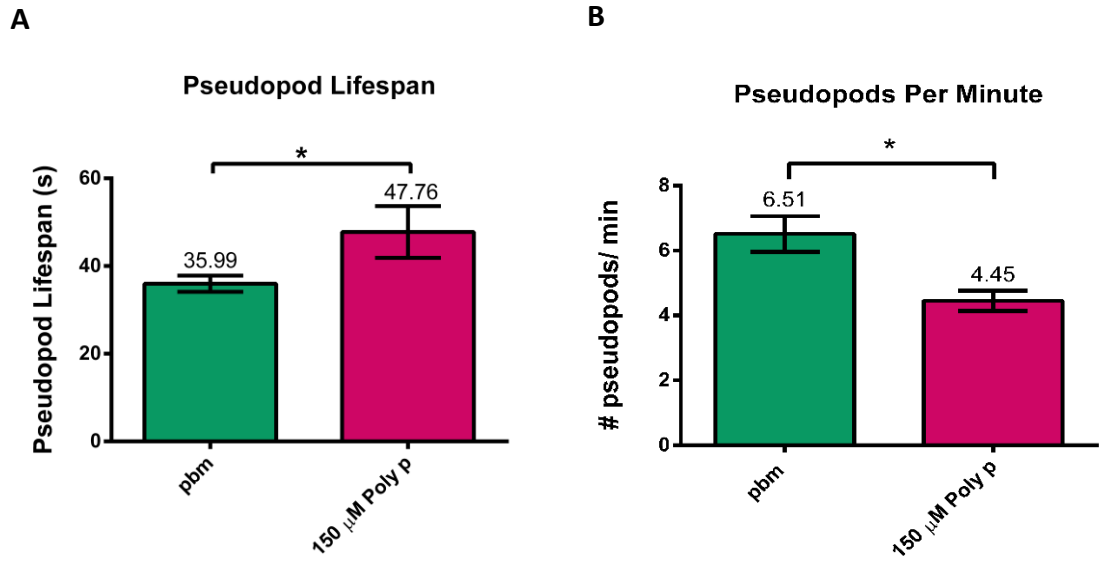

Figure 1. Polyphosphate effect on pseudopod production in D. dicsoideum (n=4)

(A) Average pseudopod lifespan over five minutes for cells treated with the control buffer pbm or 150 μM polyphosphate for 24 hours in HL5 medium. (B) The average number of pseudopods formed over five minutes was quantified for cells treated with pbm or polyphosphate for 24 hours in HL5 medium. Statistical analyses were performed as two-tail T tests. * signifies that $p < 0.05$. The mean and standard error of each experiment are plotted.

Polyphosphate increases mitochondrial size/mass irrespective of cell size and surface area

Polyphosphate is known to regulate many aspects of cell growth in *Dictyostelium* including cell proliferation, cytokinesis, cell aggregate size, and bacterial survival inside the cell. Many of these processes are already linked to important metabolic pathways. Additionally, high

concentrations of polyphosphate are linked to cell death in *Dictyostelium*. Therefore, I first examined the effect of polyphosphate on mitochondria, as they are key organelles in aerobic metabolism and certain apoptosis pathways. Mitochondrial size and mass in wild type AX2 cells were assessed by flow cytometry using 0.1 μM Mitotracker green dye, which localizes to the mitochondria and fluoresces green when excited. Higher fluorescence intensity correlates to larger mitochondria. Relative changes in cell surface area and volume were also approximated using the forward scatter (Fsc-A) measurement of cell size. I found that, compared to the control group, the mitochondria in cells treated with 150 μM polyphosphate were significantly larger. The average fluorescence intensity in cells treated with polyphosphate was 2.19 times that of the control group (figure 2). Squaring and cubing the Fsc-A measurement to approximate surface area and volume respectively indicated that polyphosphate also increases cell surface area and volume. However, the increase in mitochondrial size/mass due to polyphosphate was greater than the observed increases in cell surface area and volume. Polyphosphate increased mitochondrial size/mass by 1.99 times more than the Fsc-A size measure, 1.74 times the relative increase in surface area and 1.53 times the relative increase in volume.

Another intriguing observation from this experiment was a possible impact of polyphosphate on dye uptake. The experimental technique was refined multiple times to eliminate polyphosphate presence during the dye incubation because a fluorescence increase was observed even with very brief (<30 minutes) treatment with polyphosphate. Hypothesizing that polyphosphate may increase cellular uptake of the dye apart from its effects on the mitochondria, I tested a new system in which the polyphosphate was removed and then cellular processes halted using an ice bath before dye incubation. In the new experimental design, a smaller, or negligible after very brief treatment, fluorescence increase due to polyphosphate was observed.

Therefore, just the presence of polyphosphate appears to increase cellular uptake of dye, which is not indicative of the actual influence of polyphosphate on the mitochondrial size and mass.

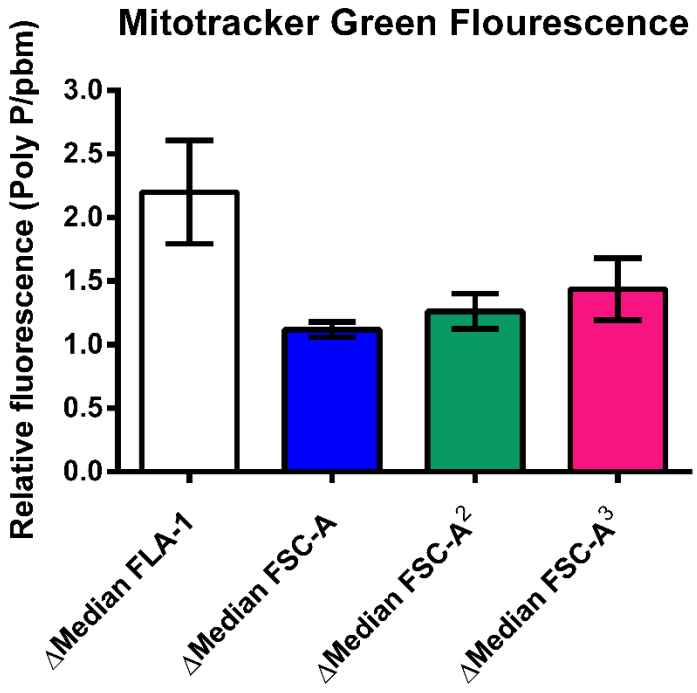

Figure 2. Effect of polyphosphate on mitochondrial size and mass in *D. discoideum* (n=4)

Fluorescence intensity was measured using a flow cytometer for at least 10,000 cells that had been treated with pbm or 150 μM polyphosphate. Cells were incubated with 0.1 μM Mitotracker green dye before fluorescence was measured. The square and cube of median FSC-A were used to approximate cell surface area and volume, respectively. Median FLA-1 is a measure of fluorescence intensity. Data was normalized to the pbm group, which had no added polyphosphate. The increase in Median FLA-1 attributed to polyphosphate was significantly higher than the increases in Median FSC-A and Median FSC-A^2 ($p < 0.05$)

Polyphosphate may increase intracellular ATP levels

Due to the observed effects of polyphosphate on mitochondrial size and mass, I next examined the effect of polyphosphate on intracellular ATP levels. Since the mitochondria are the site of the citric acid cycle, electron transport, oxidative phosphorylation, B-oxidation, and other ATP-generating processes, I hypothesized that polyphosphate would also increase intracellular ATP levels in addition to mitochondrial size and mass, A Thermofisher firefly luciferase ATP

determination kit was used to quantify intracellular ATP levels in cells treated with 150 μM polyphosphate or an equivalent volume of pbm. While the results were not significantly significant after n=4 replicates, a small possible increase in ATP levels due to polyphosphate was observed (figure 3). The luminescence of cells treated with polyphosphate was about 374 nM while that of the cells in the control group was about 292 nM. With more replicates, the results may reach significance. Measuring the response to other physiological (between 0 μM and 150 μM) concentrations of polyphosphate may also reveal whether it significantly affects ATP levels in *D. discoideum.*

Polyphosphate does not affect oxygen consumption in *D. discoideum*

Oxygen consumption was measured next, as it is an important indicator of aerobic metabolism efficiency in eukaryotes. Using a molecular probe kit from Abcam, I quantified oxygen consumption over 90 minutes in cells treated with 150 μM polyphosphate or an equivalent volume of pbm buffer. Prior to the experiment, I predicted that oxygen consumption would increase in cells treated with polyphosphate since the mitochondria enlarged in the presence of polyphosphate. However, the data indicate negligible differences in oxygen consumption between groups (figure 4).

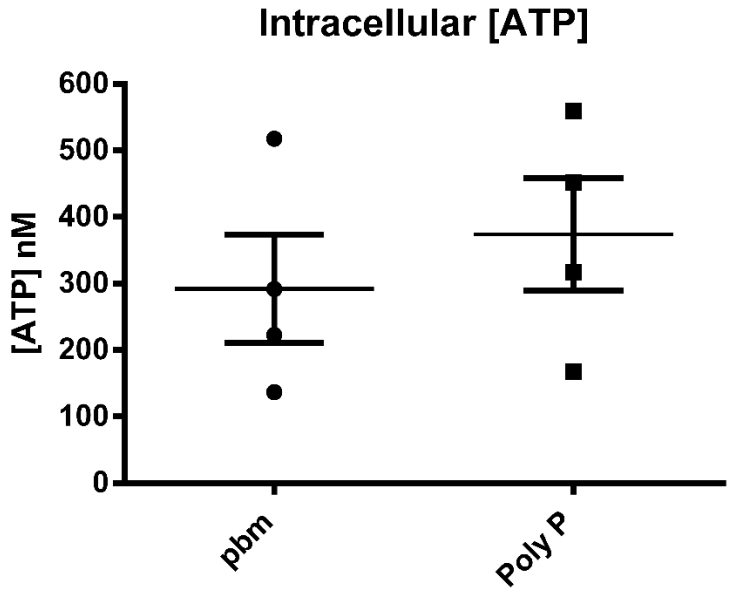

Figure 3. **Effect of polyphosphate on intracellular ATP concentration in *D. discoideum* (n=4)**

Intracellular ATP concentration was quantified using a plate reader and 96-well plate for cells that had been treated with pbm or polyphosphate for 24 hours in HL5 medium. Mean and standard error are plotted.

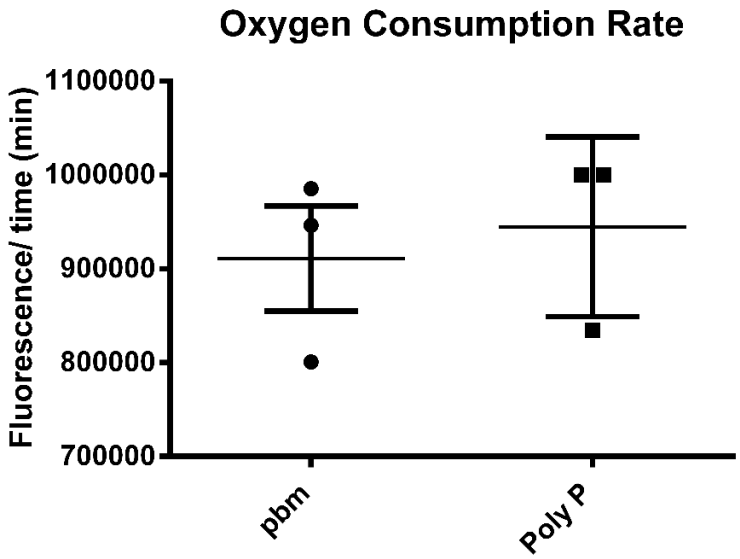

Figure 4. Effect of polyphosphate on rate of oxygen consumption in *D. discoideum* (n=4)

Oxygen consumption was measured in cells treated with pbm or 150 μM polyphosphate for 24 hours in HL5 medium. The rate was calculated from the slope of the most linear segment of the best fit line generated from fluorescence/time. Fluorescence was measured in arbitrary units by the plate reader.

Polyphosphate does not significantly affect glucose consumption in *D. discoideum*

Glucose metabolism is another essential source of energy for eukaryotic cells since glucose feeds into pathways for energy production, such as glycolysis and the TCA cycle, biosynthesis, such as the pentose phosphate pathway, and the cell cycle as an indicator of sufficient nutrients for cell division. Therefore, changes in cellular glucose levels would indicate that polyphosphate may work through a glucose-regulated metabolic pathway to enact its previously observed effects on processes like proliferation inhibition and metabolism of phagocytosed bacteria. Intracellular glucose levels were quantified with a colorimetric kit from Abcam. No significant difference in glucose concentration per cell was observed after n=4 replicates (Figure 5). Previous experiments determined that polyphosphate does increase protein content in *Dictyostelium* cells [2], so the amount of glucose per protein in cell could be lower in cells treated with polyphosphate, but further experimentation is necessary to verify this.

Polyphosphate regulates expression of key genes in *Dictyostelium*

Finally, I performed qPCR using *Dictyostelium* mRNA to determine the effect of polyphosphate on genes that are likely to be involved in polyphosphate-regulated pathways. The genes were selected by screening a variety of available mutants for reduced sensitivity to polyphosphate-induced proliferation inhibition. Among the mutants that showed reduced proliferation inhibition compared to wild type AX2 cells were plc$^-$, IplA$^-$, pten$^-$, and piaA$^-$, and grld$^-$. Thus, expression of the genes Plc (Phospholipase C), IplA (Inositol 1,4,5-trisphosphate receptor-like protein A) , Pten (Phosphatase and tensin homolog), PiaA (Protein pianissimo A), and Grld (glutamate receptor-like protein D) was targeted in this experiment. These genes encode important elements of cell signaling, growth, and biosynthesis pathways. RNA was extracted from wild type AX2 cells in shaking culture after 0, 4, 8, and 12 hours of exposure to

polyphosphate or the control buffer pbm. The qPCR results show that polyphosphate increases expression of Plc, IplA, Pten, and PiaA at all time points while decreasing expression of Grld at all points compared to the control group (figure 6).

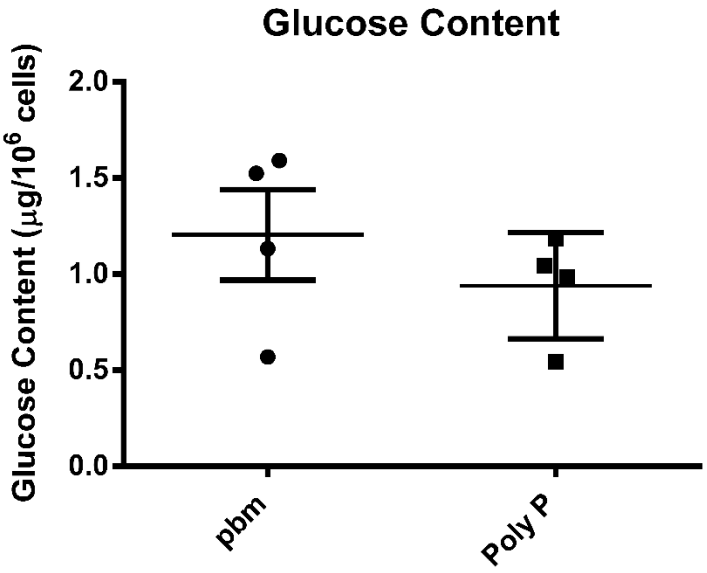

Figure 5. Effect of polyphosphate on intracellular glucose content in *D. discoideum* (n=4)

Glucose content was measured using a plate reader in cells that had been treated with pbm or 150 μM polyphosphate for 24 hours in HL5 medium. Mean and standard error are plotted.

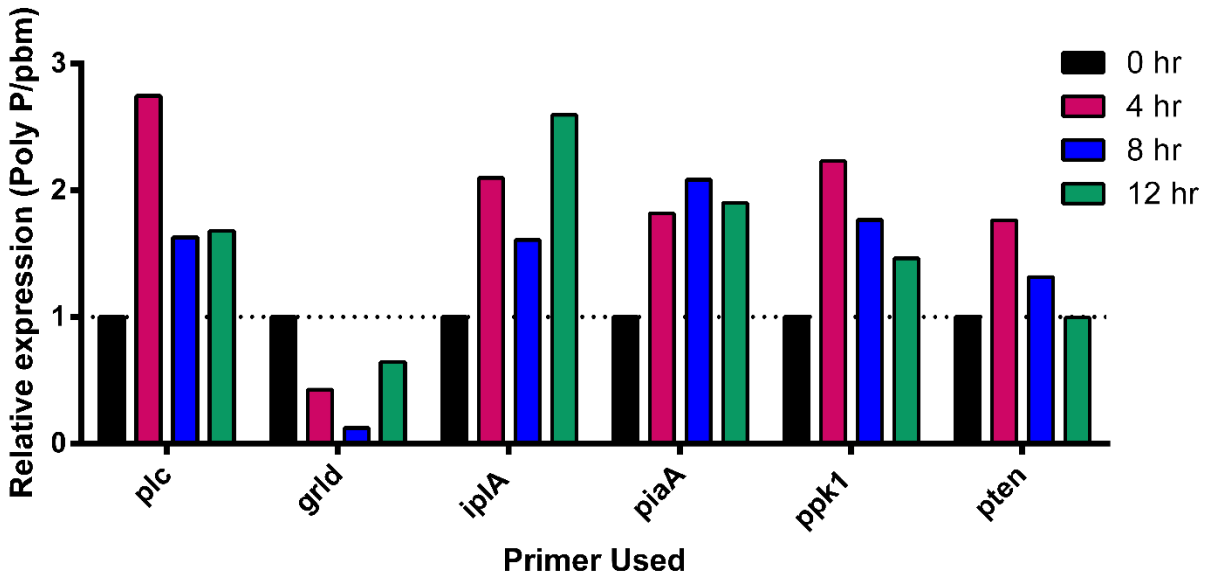

Figure 6. Effect of Polyphosphate on expression of Plc, Grld, IplA, PiaA, Ppk1, and Pten in *Dictyostelium*

qPCR was formed using cDNA made with mRNA extracted from *Dictyostelium* cells after exposure to pbm or 150 µM polyphosphate at 0, 4, 8, or 12 hours. The graph depicts the ratio of polyphosphate treatment gene expression to control group gene expression after normalization to t= 0 hr.

SECTION IV

CONCLUSION

Polyphosphate may hinder macrophage efficacy through impeding pseudopod production

One possible explanation for why pseudopod production decreases in *Dictyostelium* cells treated with polyphosphate is that polyphosphate may inhibit the cells' ability to manipulate their cytoskeletons. For instance, the cells may struggle forming the pseudopods, hence the decreased number of pseudopods formed per minute, and may also have trouble retracting the pseudopods back into the cell body, hence the increased pseudopod lifespan. In one study, tumor cells lacking the human exopolyphosphatase H-prune demonstrated increased motility and metastasis [7]. Pseudopod formation is essential for macrophage activity since pseudopods are the mechanisms by which they phagocytose molecules or microbes and travel to target destinations [18]. Therefore, added polyphosphate may decrease the overall motility of *Dictyostelium* and other macrophage type cells. Manipulation of actin filaments, which *Dictyostelium* cells use for pseudopod production and migration, has also been associated with polyphosphate production [19], so it is possible that extracellular polyphosphate may interfere with actin manipulation to cause the observed defects in pseudopod production. Finally, the necessity of pseudopod formation in both the uptake of microbes and in directing them to intracellular compartments for digestion [18] may be one reason behind the necessity for polyphosphate in bacterial survival inside macrophage-like cells.

The mitochondria may be a means of polyphosphate regulation of cellular energetics and metabolism

In the process of facilitating aerobic respiration, mitochondria produce the majority of healthy cells' energy in the form of ATP but produce potentially damaging reactive oxygen species such as super-oxides and peroxides in the process. Since it is essential that the mitochondria keep these ROS sequestered away from the rest of the cell, many apoptosis cascades are triggered by mitochondrial damage and ROS escape [20]. ROS also play a role in creating an inhospitable environment for intracellular pathogens, especially in the cellular compartments in which bacterial invaders are destroyed [21]. Unsurprisingly, polyphosphate is one means by which bacteria can survive oxidative stress due to ROS [22], which may help explain the increase in mitochondrial size/mass in *Dictyostelium* cells due to added polyphosphate. Unfortunately, little is known about the effect of polyphosphate on ROS in mammalian cells.

Added polyphosphate also increases intracellular and extracellular ATP levels in human cell lines like HUVEC (human umbilical vein endothelial cells) and osteosarcoma cells [23, 24]. This prospect is promising for further experimentation into ATP levels in *Dictyostelium* cells. Additionally, it suggests that polyphosphate may contribute to fuel stores in bioenergetic processes, which makes sense especially in the context of polyphosphate acting as a pre-starvation factor in *Dictyostelium* cells.

Alteration of gene expression patterns provides insight into polyphosphate regulatory pathway components

Interestingly, polyphosphate increased expression of the three calcium signaling pathways genes Pten, Plc, and IplA as well as that of the G protein-coupled receptor associated gene PiaA. These results suggest that polyphosphate may be involved in this calcium signaling pathway and that the increased expression of its genes may enable polyphosphate to enact its

related effects. Conversely, expression of the putative G protein-coupled receptor Grld is downregulated by physiological (150 μM) levels of polyphosphate. It is possible that expression of the receptor, as is has been shown to mediate sensing of polyphosphate in *Dictyostelium* [14] also functions through a negative feedback loop in order to prevent cell damage, as high concentrations of polyphosphate can cause cell death [25].

Further experimentation will focus on determining whether a significant increase in ATP levels in *Dictyostelium* cells can be attributed to polyphosphate and assessing the effects of polyphosphate on extracellular media acidification as a measure of glycolysis. I will also examine the possible effects of polyphosphate on membrane permeability given my observations in the mitochondrial assays and the connection of polyphosphate to vascular hyperpermeability in mammals [9].

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